Modeling chemotaxis reveals the role of reversed phosphotransfer and a bifunctional kinase-phosphatase

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Modeling Chemotaxis Reveals the Role of Reversed Phosphotransfer and a Bi-Functional Kinase-Phosphatase

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Abstract

Understanding how multiple signals are integrated in living cells to produce a balanced response is a major challenge in biology. Two-component signal transduction pathways, such as bacterial chemotaxis, comprise histidine protein kinases (HPKs) and response regulators (RRs). These are used to sense and respond to changes in the environment. *Rhodobacter sphaeroides* has a complex chemosensory network with two signaling clusters, each containing a HPK, CheA. Here we demonstrate, using a mathematical model, how the outputs of the two signaling clusters may be integrated. We use our mathematical model supported by experimental data to predict that: (1) the main RR controlling flagellar rotation, CheY, aided by its specific phosphatase, the bifunctional kinase CheA3, acts as a phosphate sink for the other RRs; and (2) a phosphorelay pathway involving CheB2 connects the cytoplasmic cluster kinase CheA3 with the polar localised kinase CheA2, and allows CheA2-P to phosphorylate non-cognate chemotaxis RRs. These two mechanisms enable the bifunctional kinase/phosphatase activity of CheA3 to integrate and tune the sensory output of each signaling cluster to produce a balanced response. The signal integration mechanisms identified here may be widely used by other bacteria, since like *R. sphaeroides*, over 50% of chemotactic bacteria have multiple cheA homologues and need to integrate signals from different sources.

Introduction

Two-component signaling pathways are the major mechanism by which bacterial cells sense and respond to changes in their environment. They regulate processes as diverse as virulence, gene expression, development and motility [1]. Bacteria can have over 100 different two-component pathways per cell, one form of which controls swimming behavior. This chemosensory pathway has been extensively studied as an example of a two-component signaling pathway as it provides a model of signaling, signal termination and receptor adaptation. Mathematical modeling has proved particularly useful in helping to understand the complexity of *Escherichia coli* chemotaxis [2–10].

Most chemotactic bacteria sense changes in their extracellular environment using transmembrane chemoreceptors [11]. These chemoreceptors signal via an intracellular signaling cascade to the flagellar motor. In the case of *E. coli*, the signaling cascade is well understood [12,13]. The chemoreceptors form a quaternary complex at the cell poles with the scaffold protein CheW and the histidine protein kinase, CheA [14–16]. The chemoreceptors detect changes in the periplasmic chemoeffect concentration and control the rate at which CheA autophosphorylates on a conserved histidine residue. In response to decreased attractant concentration, the chemoreceptors signal to increase the rate of CheA autophosphorylation [17–19]. Following autophosphorylation, the phosphoryl group is transferred from the histidine residue of CheA to an aspartate residue in one of the two response regulators (RRs), CheY or CheB [20–22]. CheY-P is released from the chemotaxis cluster and diffuses through the cell to the flagellar motor. CheY-P binds the FliM component of the flagellar motors, causing the direction of flagellar rotation to switch from counter-clockwise to clockwise resulting in tumbling of the bacterium [23,24]. CheA-P also phosphorylates the methylesterase CheB, which facilitates adaptation of the chemoreceptor cluster [25,26]. CheY-P and CheB-P both naturally auto-dephosphorylate [27], although the rate of CheY-P dephosphorylation is enhanced by CheZ to allow signal termination within the time required for effective gradient sensing [28,29].

In contrast to *E. coli*, *Rhodobacter sphaeroides* has a more complex signaling pathway with multiple copies of the signaling proteins encoded by three major chemosensory operons [30]. Many other bacterial species appear to have multiple chemosensory operons as analysis of sequenced genomes suggests that ~50% of species with any *che* genes have at least two *cheA*s [30–32]. This raises the question of how behavior is controlled by two or more homologous pathways and how sensory data from each of the
Author Summary

Chemotactic bacteria sense nutrient gradients and swim towards better environments for growth. A cluster of receptors in the cell membrane detects nutrient levels and signals via a cytoplasmic signaling pathway to the flagellum. The complexity of this signaling pathway varies across different bacterial species. The relatively simple pathway used by Escherichia coli is well understood; however, many bacteria, for example Rhodobacter sphaeroides, have more sophisticated pathways that, as well as being able to detect nutrients, are also able to assess the metabolic state of the cell. The receptors that detect metabolic state are located within an additional cluster that is physically distinct from the one that senses nutrients. In this work, we use a combination of experimentation and mathematical modeling to gain insight into the complex decision-making mechanisms that enable bacteria to weigh-up different stimuli and decide upon an appropriate response. We find novel communication mechanisms between the two signaling clusters that allow the outputs of the signaling pathways to be balanced and tuned according to the prevailing environmental conditions. The signaling principles identified here are likely to be used in other complex sensory networks.

pathways are integrated to produce a balanced response. Under laboratory conditions, R. sphaeroides swims using a single sub-polar unidirectional flagellum (Fla1), which is controlled by the protein products of cheOp2 and cheOp3 [33–38]. The intracellular signaling cascade controlling the Fla1 flagellum comprises three CheA kinase proteins (denoted CheA2, CheA3, CheA4), three CheY proteins (CheY5, CheY4 and CheY6), and two CheBs (CheB1, CheB2) [34,35,39–42]. CheA3 and CheA4 are unusual CheAs in that they lack some of the domains found in E. coli CheA and neither protein is capable of autophosphorylation [43]. However, together CheA3 and CheA4 have all of the activities of a functional CheA with CheA4 forming a homodimer that binds ATP and phosphorylates the Hpt domain of CheA3.

The signal transduction proteins are organized and localized into two distinct sensory clusters and the signaling output of both clusters is required for chemotaxis [43,44]. CheA2 is located in a chemotaxis cluster at the cell poles, which comprises transmembrane chemoreceptors and the signal transduction proteins encoded by cheOp2 [44]. This cluster detects changes in the periplasmic concentration of chemoeffectors. Previous data show that CheA2-P rapidly phosphorylates CheY5, CheY4, CheY6, CheB1, and CheB2 (Figure 1), although the kinetics of phosphotransfer differ in each case [45]. CheA3 and CheA4 localize to a second chemotaxis cluster found in the cytoplasm [44]. This cluster contains the signal transduction proteins encoded by cheOp3 along with the soluble chemoreceptors and is believed to sense the metabolic state of the cell [44,46]. CheA3-P rapidly phosphorylates only the RRs, CheY6 and CheB2 [43,47]. In addition, CheA3 has an aspartyl-phosphate phosphatase activity that is specific for CheY6-P; this activity is required for the rapid signal termination that is necessary for chemotactic responses [48]. CheA3 in conjunction with CheA4 can therefore be considered to be a bifunctional kinase/phosphatase.

In vitro studies have shown that all of the R. sphaeroides CheYs can bind the flagellar switch protein, FliM, and that this binding is strongest when the CheYs are phosphorylated [49], but less is known about the effect of CheY/CheY-P binding to FliM on flagellar rotation. CheY6 is essential for chemotaxis and CheY6-P

Figure 1. Diagram showing the RRs phosphorylated by the polar (blue) and cytoplasmic (red) chemotaxis clusters. The polar chemotaxis cluster contains CheA2 and responds to the external environment. CheA2 autophosphorylates and can then serve as a phosphodonor for all of the RRs. The cytoplasmic chemotaxis cluster contains CheA3 and CheA4, and is thought to respond to the metabolic state of the cell. CheA3 is phosphorylated by CheA4. Unlike CheA2-P, CheA3-P is not able to phosphorylate all of the RRs – it can only phosphorylate CheY6 and CheB2. All of the RR-Ps spontaneously autodephosphorylate; however, the dephosphorylation of CheY6-P is accelerated by the phosphatase activity of CheA4.

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alone is capable of causing the chemotactic stop that is necessary for changing swimming direction [39]. However, CheY6 alone cannot support chemotaxis; either CheY3 or CheY4 are also required. Furthermore, phosphorylation site mutants of CheY3, CheY4 and CheY6 do not support chemotaxis [39], suggesting that phosphorylation of all of these CheYs is necessary for chemotaxis.

Thus there are two complete chemosensory pathways in R. sphaeroides, localized to different regions of the cell and with different patterns and kinetics of phosphotransfer to the RRs. However, the outputs of these two signaling pathways must be
integrated to control the behavior of a single flagellar motor. In vivo biochemistry identified which RRs are phosphorylated by each CheA and the kinetics of the interactions, however, assessing the relative contribution made to RR-P levels by each of these CheAs in vivo is more complex, since all of the RR-Ps will be competing with one another for phosphorylation by the CheAs. We used mathematical modeling to predict the possible signaling pathways within this complex system and tested these predictions experimentally.

The aim of this study was therefore to combine our knowledge of the kinetic preferences of the signaling reactions gained from in vivo biochemistry with the in vivo data on protein copy number within a mathematical model that can predict the changes in RR-P levels resulting from changes in CheA activity at either cluster. This model was then used to analyze the contribution made by each cluster in controlling RR-P levels and the dynamics of the signaling reactions. Using the model, we identified unexpected key roles for reversed phosphotransfer between RR-P and CheA in the network, which would enable communication between the two sensory clusters and thus regulate the output signals. In addition, we demonstrated that the principal RR, CheY6, with the aid of its specific phosphatase, the bifunctional CheA2/CheA4 kinase, could act as a phosphate sink for the other RR-Ps. Regulation of the output of sensory networks by the activity of key kinase/phosphatase proteins is likely to be a common mechanism, but this is one of the first to be identified that balances the outputs of two interconnected pathways.

Results

Construction of the mathematical model

Within an R. sphaeroides cell, CheA2 has been shown to localize to the polar chemotaxis cluster, while CheA3 and CheA4 localize to the cytoplasmic cluster [44]. All the R. sphaeroides CheYs are free to diffuse throughout the cytoplasm of the cell enabling communication between the receptor clusters and flagellar motor. Unlike E. coli, the CheBs are also diffuse in the cytoplasm [39,44].

As illustrated in Figure 1, CheA2-P can phosphorylate all of the RRs, whilst CheA7-P is only able to phosphorylate CheY6 and CheB2. What is the reason for this discrimination and how does it contribute towards the chemotactic response of the cell? To understand the role of each signaling cluster we constructed an ordinary differential equation (ODE) model of an R. sphaeroides cell as detailed in Text S1. The model integrates in vivo protein expression levels with in vivo data on the kinetic preference of the CheAs for each of the RRs to predict RR-P levels throughout a simulated chemotactic response. The model includes the phosphorylation reactions shown in Table 1 and was parameterized with published reaction rate constants and protein expression levels (Table 2).

The parameters for the phosphotransfer reactions were obtained by parameter fitting the previously published R. sphaeroides chemotaxis phosphotransfer assay data [43,45,48], where CheA3-P served as a phosphodonor to the RRs (Table 3). In the few cases where the assays were not very sensitive to the rate of reversed phosphotransfer from RR-P to CheA, reliable estimates of these rates were obtained using alternative phosphotransfer assays, in which RR-P was mixed with unphosphorylated CheA2 or CheA3 (examples shown in Figure 2). In these reactions, RR-P was generated using purified phosphorylated CheA P1 domains (either CheA2P1-P or CheA3P1-P) as the phosphodonor; control reactions lacking RR showed no phosphotransfer from CheA3P1-P or CheA2P1-P to either CheA2 or CheA3. These experiments showed that while CheA2 is phosphorylated by CheB2-P (Figure 2C) it is not phosphorylated by CheY6-P (Figure 2B). The parameter values obtained from these phosphotransfer reactions were then used in constructing the model.

Response regulator dephosphorylation rates show CheY6 acts as a phosphate sink

R. sphaeroides responds to brief stimuli, returning to prestimulus behavior in less than 1 s [50]. This requires a rapid rate of signal termination. The measured autodephosphorylation half-times of the chemotaxis RRs, however, vary from ~4 s for CheY6-P to ~4000 s for CheB1-P (Table 4). As R. sphaeroides does not have a CheZ homologue, an alternative dephosphorylation mechanism is required. Recently, CheA3 was shown to be a specific phosphatase for CheY6-P [48], but no phosphatases have been identified for the remaining chemotaxis RRs.

Phosphate sinks have been shown to be involved in signal termination in several bacterial signaling pathways [51–54]. To test whether a similar mechanism operates in R. sphaeroides, we used the model to predict the decay timecourse of RR-P levels resulting from simultaneously switching off autodephosphorylation of CheA2 (reaction 1 in Table 1) and the phosphorylation of CheA3 by CheA4 (reaction 2 in Table 1). Although, the model incorporates the experimentally determined autodephosphorylation rates (reactions 10–15b of Table 1), interestingly, the model predicts that levels of all of the RR-Ps decay with half-lives shorter than ~7 s (Table 4), which is much faster than their experimen-
CheY6 greatly increased the simulation half-lives of the remaining RR-Ps. We found that only the removal of RR-Ps (Table 4), suggesting that CheY6 acts as a phosphate sink only CheY6-P has a phosphatase. The only route by which the tally measured autodephosphorylation rates. Within the model, only CheY6-P has a phosphatase. The only route by which the model could predict dephosphorylation rates for the other RR-Ps that are faster than their autodephosphorylation rates is for one or more of the RR-Ps to be acting as “phosphate sinks”, with the dephosphorylation of the target RR-P proceeding via reversed phosphotransfer to a CheA, which in turn transfers the phosphoryl group to the sink RR.

To determine which RR-Ps could act as sinks, we simulated RR-P decay rates in cells deleted for a single RR e.g. for the cell lacking CheY4 we changed $k_{14}$ to zero and measured the simulation half-times (Table 4). This is the result of significant quantities of CheA3-P and CheA2-P being present at steady state, and persisting for some time after the autophosphorylation reactions (reactions 1 and 2) were turned off. This allows levels of RR-P to be replenished resulting in a RR-P simulation half-time that is slower than their autodephosphorylation half-times. Interestingly, even in the absence of CheY6, the other RRs (CheY3, CheY4 and CheB2) may act as phosphate sinks for CheB1-P i.e. CheB1-P acts as a phosphodonor for CheA2 which in turn donates the phosphoryl group to the sink RR.

Table 2. Parameter values directly determined from experimental data.

<table>
<thead>
<tr>
<th>Rate</th>
<th>Description</th>
<th>Value</th>
<th>Standard error</th>
<th>Units</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{1}$</td>
<td>CheA2 autophosphorylation</td>
<td>0.12</td>
<td>0.02</td>
<td>$s^{-1}$</td>
<td>[45]</td>
</tr>
<tr>
<td>$k_{2}$</td>
<td>Phosphorylation of CheA2 by CheA4</td>
<td>0.98</td>
<td>0.17</td>
<td>$s^{-1}$</td>
<td>[43]</td>
</tr>
<tr>
<td>$k_{10}$</td>
<td>CheY6P autodephosphorylation</td>
<td>$1.93 \times 10^{-2}$</td>
<td>$0.20 \times 10^{-2}$</td>
<td>$s^{-1}$</td>
<td>[43]</td>
</tr>
<tr>
<td>$k_{11}$</td>
<td>CheY4 autophosphorylation</td>
<td>$1.82 \times 10^{-2}$</td>
<td>$0.13 \times 10^{-2}$</td>
<td>$s^{-1}$</td>
<td>[43]</td>
</tr>
<tr>
<td>$k_{12}$</td>
<td>CheY6 autophosphorylation</td>
<td>$1.69 \times 10^{-1}$</td>
<td>$0.12 \times 10^{-1}$</td>
<td>$s^{-1}$</td>
<td>[43]</td>
</tr>
<tr>
<td>$k_{13}$</td>
<td>CheB2P autophosphorylation</td>
<td>$1.73 \times 10^{-4}$</td>
<td>$0.06 \times 10^{-4}$</td>
<td>$s^{-1}$</td>
<td>[43]</td>
</tr>
<tr>
<td>$k_{14}$</td>
<td>CheB1P autophosphorylation</td>
<td>$1.33 \times 10^{-2}$</td>
<td>$0.12 \times 10^{-2}$</td>
<td>$s^{-1}$</td>
<td>[43]</td>
</tr>
<tr>
<td>$k_{15b}$</td>
<td>CheY6P dephosphorylation by CheA2</td>
<td>$5.20 \times 10^3$</td>
<td>$0.32 \times 10^3$</td>
<td>$(Ms)^{-1}$</td>
<td>[44]</td>
</tr>
<tr>
<td>$k_{15b}$</td>
<td>CheY4P dephosphorylation by CheA3</td>
<td>$5.20 \times 10^3$</td>
<td>$0.32 \times 10^3$</td>
<td>$(Ms)^{-1}$</td>
<td>[44]</td>
</tr>
</tbody>
</table>

Total concentration of CheA2 | 89.9 | 7.6 | $\mu$M | [69] |
Total concentration of CheA3 | 89.9 | 10.4 | $\mu$M | [69] |
Total concentration of CheY3 | 3.5 | 1.0 | $\mu$M | [68] |
Total concentration of CheY4 | 13.8 | 2.8 | $\mu$M | [68] |
Total concentration of CheY6 | 225 | 27 | $\mu$M | [68] |
Total concentration of CheB1 | 81.2 | 3.8 | $\mu$M | [69] |
Total concentration of CheB2 | 20.8 | 2.1 | $\mu$M | [69] |

Table 3. Parameter values estimated indirectly by fitting to phosphotransfer reaction data.

<table>
<thead>
<tr>
<th>Rate</th>
<th>Description</th>
<th>Value</th>
<th>Units</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{3}$</td>
<td>CheA2P to CheY4 phosphotransfer</td>
<td>$6.60 \times 10^3$</td>
<td>$(Ms)^{-1}$</td>
<td>This study using [45]</td>
</tr>
<tr>
<td>$k_{3}^{-1}$</td>
<td>CheA2P to CheY4 reverse phosphotransfer</td>
<td>$1.17 \times 10^3$</td>
<td>$(Ms)^{-1}$</td>
<td>This study using [45]</td>
</tr>
<tr>
<td>$k_{4}$</td>
<td>CheA4P to CheY4 phosphotransfer</td>
<td>$8.85 \times 10^3$</td>
<td>$(Ms)^{-1}$</td>
<td>This study using [45]</td>
</tr>
<tr>
<td>$k_{4}^{-1}$</td>
<td>CheA4P to CheY4 reverse phosphotransfer</td>
<td>$2.32 \times 10^3$</td>
<td>$(Ms)^{-1}$</td>
<td>This study using [45]</td>
</tr>
<tr>
<td>$k_{5}$</td>
<td>CheA2P to CheY4 phosphotransfer</td>
<td>$1.54 \times 10^3$</td>
<td>$(Ms)^{-1}$</td>
<td>This study using [45]</td>
</tr>
<tr>
<td>$k_{5}^{-1}$</td>
<td>CheA2P to CheY4 reverse phosphotransfer</td>
<td>0</td>
<td>$(Ms)^{-1}$</td>
<td>This study</td>
</tr>
<tr>
<td>$k_{6}$</td>
<td>CheA2P to CheB1 phosphotransfer</td>
<td>$1.78 \times 10^3$</td>
<td>$(Ms)^{-1}$</td>
<td>This study using [45]</td>
</tr>
<tr>
<td>$k_{6}^{-1}$</td>
<td>CheA2P to CheB1 reverse phosphotransfer</td>
<td>$2.85 \times 10^3$</td>
<td>$(Ms)^{-1}$</td>
<td>This study using [45]</td>
</tr>
<tr>
<td>$k_{7}$</td>
<td>CheA2P to CheB2 phosphotransfer</td>
<td>$3.07 \times 10^3$</td>
<td>$(Ms)^{-1}$</td>
<td>This study using [45]</td>
</tr>
<tr>
<td>$k_{7}^{-1}$</td>
<td>CheA2P to CheB2 reverse phosphotransfer</td>
<td>$1.53 \times 10^3$</td>
<td>$(Ms)^{-1}$</td>
<td>This study</td>
</tr>
<tr>
<td>$k_{8}$</td>
<td>CheA2P to CheY6 phosphotransfer</td>
<td>$7.75 \times 10^3$</td>
<td>$(Ms)^{-1}$</td>
<td>This study using [45]</td>
</tr>
<tr>
<td>$k_{8}^{-1}$</td>
<td>CheA2P to CheY6 reverse phosphotransfer</td>
<td>$2.83 \times 10^3$</td>
<td>$(Ms)^{-1}$</td>
<td>This study using [45]</td>
</tr>
<tr>
<td>$k_{9}$</td>
<td>CheA2P to CheB2 phosphotransfer</td>
<td>$6.15 \times 10^3$</td>
<td>$(Ms)^{-1}$</td>
<td>This study using [45]</td>
</tr>
<tr>
<td>$k_{9}^{-1}$</td>
<td>CheA2P to CheB2 reverse phosphotransfer</td>
<td>$3.10 \times 10^3$</td>
<td>$(Ms)^{-1}$</td>
<td>This study using [45]</td>
</tr>
</tbody>
</table>

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that the simulated dephosphorylation half-time for CheB1-P was 30 minutes.

CheB1.

one or more of other RRs therefore act as phosphate sinks for CheA2. The signal termination times for all of the RR-Ps would be longer without the phosphatase (Table 4), as the model also predicted that the signal termination times for all of the RR-Ps (Table 5), with phosphorylation levels of the total chemotaxis RR pool rising from ~55% to ~97%. This was the result of increased levels of CheY6-P (due to decreased dephosphorylation) leading to higher CheA2-P and CheA3-P concentrations and therefore higher levels of the other RR-Ps. The model also predicted that the signal termination times for all of the RR-Ps would be longer without the phosphatase (Table 4), as CheY6 would be less effective as a phosphate sink for the other RR-Ps. The model therefore highlights the importance of the phosphatase activity in CheA3, and demonstrates that although it is specific for CheY6-P, the phosphatase activity indirectly affects the concentration of the other RR-Ps and their signal termination rates as outlined above (Figure 3A). Removal of the phosphatase activity is therefore predicted to cause a general increase in RR-P levels, which could account for the non-chemotactic phenotype of the strains lacking phosphatase activity [40].

The phosphatase activity of CheA3 is required for CheY6 to work as an efficient phosphate sink.

In addition to containing the Hpt domain needed for phosphorylation of CheY6, CheA3 is also a phosphatase specific for CheY6-P. This phosphatase activity has previously been shown to be essential for chemotaxis [48]. We used the model to determine the effect of phosphatase removal on RR-P levels and signal termination times, by setting the rate constants for the CheA3 phosphatase reactions (15a) and (15b) in Table 2 to zero.

Analysis, where we varied each parameter by factors of 0.1, 0.5, 1.5 and 10, and measured the effect on the simulation half-life of CheB1-P (Table S1). For the parameters that were determined experimentally, the standard error lies well within the range covered by factors of 0.5 and 1.5. The simulation half-life of CheB1-P was robust to large changes in the majority of parameters, and in all cases remained much faster than the CheB1-P autodephosphorylation rate, but as would be expected showed some sensitivity towards those parameters directly involved in the operation of the CheY6 phosphate sink i.e. the rates of phosphotransfer between CheB1 and CheA3 and between CheA2 and CheY6. In addition, the system was also sensitive to large changes, well outside the range covered by experimental error in parameter determination, in the total concentrations of CheA2 and CheY6. This sensitivity analysis indicates that the parameter space in which the phosphate sink mechanism will work efficiently is broad and extends well beyond the range of experimental errors in the parameters themselves, suggesting that this pathway is likely to operate in vivo.

In summary, these simulated data suggest that not only is CheY6 a key regulator of flagellar motor rotation in R. sphaeroides, but it also acts as a “phosphate sink” ensuring rapid dephosphorylation of the other chemotaxis RRs (Figure 3A). This is very different from the S. meliloti sink where the sink CheY does not bind to the flagellar motor.

A phosphorelay pathway connects both chemotaxis clusters.

We modeled the consequences of chemoeffector stimulation of either of the two chemotaxis clusters by either (i) turning off CheA2 autophosphorylation (reaction 1) – parameter $k_1$ set to zero to mimic attractant stimulation of the polar chemotaxis cluster or (ii) turning off the phosphorylation of CheA2 by CheY6 (reaction 2) – parameter $k_2$ set to zero to mimic attractant stimulation of the cytoplasmic chemotaxis cluster (Figure 4). As expected, when CheA2 autophosphorylation was turned off (case (i)) there was a reduction in the phosphorylation levels of each of the RRs (Figure 4) because CheA2-P serves as a phosphodonor for all of the RRs. However, counter-intuitively, significant levels of all RR-Ps were not observed in the absence of CheY6.
remained, including CheY3-P, CheY4-P and CheB1-P, which can only be generated by CheA2-P.

Analysis of the modeling results from case (i) revealed that even though CheA2 autophosphorylation had been turned off, significant levels of CheA2-P were present and were being generated by the reversal of reaction (7) (Table 1) i.e. CheB2-P was acting as a phosphodonor for CheA2 (as demonstrated in Figure 2C). In this case, CheY6 and CheB2 were phosphorylated by CheA3-P; CheB2-P then served as a phosphodonor for CheA2 i.e. CheB2 transfers phosphoryl groups between CheA3-P and CheA2. The CheA2-P generated in this way could then phosphorylate CheY3, CheY4 and CheB1. This result suggests that CheA3-P is linked to the RRs, CheY3, CheY4 and CheB1, via a multistep phosphorelay i.e. CheA3-P (His) to CheB2 (Asp) to CheA2 (His) to CheY3/CheY4/CheB1 (Asp).

In case (ii), switching off the phosphorylation of CheA3 by CheA4 caused a reduction not only in the levels of CheY6-P and CheB2-P, but also in levels of CheY3-P, CheY4-P and CheB1-P. Levels of CheY3-P, CheY4-P and CheB1-P were affected because (a) they were dephosphorylated faster since the reduction in CheY6-P levels was accompanied by an increase in the unphosphorylated CheY6 levels which acts as a phosphate sink and (b) there was no input of phosphoryl groups at the cytoplasmic cluster for CheB2 to transfer to these RRs via CheA2. These results indicate that even when CheA2 autophosphorylation is occurring, the bifunctional kinase/phosphatase in the cytoplasmic chemotaxis cluster makes a significant contribution to the phosphorylation levels of all of the RRs.

We performed a sensitivity analysis to look at the effect of varying each of the model parameters on levels of CheY4-P when CheA2

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**Table 4. Comparison of RR-P autodephosphorylation rates with the RR-P dephosphorylation half-times predicted by the model.**

<table>
<thead>
<tr>
<th>Autodephosphorylation half-time (s)</th>
<th>Wild type cells</th>
<th>ΔcheY3</th>
<th>ΔcheY4</th>
<th>ΔcheY6</th>
<th>ΔcheB1</th>
<th>ΔcheB2</th>
<th>No phosphatase</th>
</tr>
</thead>
<tbody>
<tr>
<td>CheY1-P</td>
<td>36 ± 3</td>
<td>4.9</td>
<td>n/a</td>
<td>4.7</td>
<td>296</td>
<td>3.7</td>
<td>5.2</td>
</tr>
<tr>
<td>CheY2-P</td>
<td>38 ± 3</td>
<td>7.3</td>
<td>7.3</td>
<td>n/a</td>
<td>543</td>
<td>5.1</td>
<td>8.1</td>
</tr>
<tr>
<td>CheY3-P</td>
<td>4.1 ± 0.3</td>
<td>1.3</td>
<td>1.3</td>
<td>1.4</td>
<td>n/a</td>
<td>1.3</td>
<td>1.3</td>
</tr>
<tr>
<td>CheB1-P</td>
<td>4046 ± 150</td>
<td>4.2</td>
<td>4.2</td>
<td>4.0</td>
<td>309</td>
<td>n/a</td>
<td>4.5</td>
</tr>
<tr>
<td>CheB2-P</td>
<td>52 ± 4</td>
<td>4.8</td>
<td>4.9</td>
<td>4.8</td>
<td>430</td>
<td>4.1</td>
<td>n/a</td>
</tr>
</tbody>
</table>

*These values were calculated from the experimentally determined in vitro autodephosphorylation rate constants [48].

The model was allowed to reach a steady state where CheA3 autophosphorylation (reaction 1) and the phosphorylation of CheA2 by CheA4 (reaction 2) were both active. Then reactions 1 and 2 were turned off. These half-times represent the time taken for levels of each of the RR-Ps to fall to half of their steady state values.

Deletion of RRs was simulated by setting their total concentration in the model to zero, e.g. for ΔcheY6, Y6T = 0 (Table 2).

Lack of CheA3 phosphatase activity was simulated by setting k15a = k15b = 0 (Table 2).  

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autophosphorylation is turned off; under these conditions CheY4-P levels give a measure of the extent to which the phosphorelay is occurring (Table S2). The system was robust to changes in many of the parameters although as would be expected was sensitive to changes in parameters that directly affect either i) the rate of entry or exit of phosphoryl groups from the system e.g. rate of phosphor-
avlation of CheA3 by CheA4, rate of CheY6-P dephosphorylation (autodephosphorylation and phosphatase assisted), and the expres-
sion levels of CheA3 and CheY6 or ii) the functioning of the phosphorelay e.g. the expression levels of CheA2 and CheB2, rates of phosphotransfer between CheA3 and CheB2, between CheB2 and CheA2, and between CheA2 and CheY4. However, despite this sensitivity in almost all cases at least some phosphorylation of CheY4 was predicted indicating that the phosphorelay remained operational. In the two remaining extreme cases, where CheY6 expression levels were ten times higher than usual or where the rate of phosphorylation of CheA3 by CheA4 was ten-fold lower than the measured rate, levels of all RR-Ps, not just CheY4-P, were extremely low. These results indicate that the phosphorelay operates over a broad range of parameter space, although the extent to which it operates is sensitive to large changes in some of the parameters.

**Discussion**

The experimental work leading up to this study produced an outline architecture of the complex signaling network controlling *R. sphaeroides* chemotaxis [30]. However, the mechanism of integrating the signals produced by each of the signaling clusters to control the flagellar motor was unclear. Mathematical modeling has provided considerable insight into the probable functioning of simpler chemotaxis pathways [2,5,55], and a control engineering approach has recently been used in *R. sphaeroides* to discriminate between several possible mechanisms of CheY control of the flagellar motor [56]. In this study, a mathematical model of *R. sphaeroides* chemotaxis was formulated that integrates *in vivo* and *in vitro* biochemical data on the kinetic preferences of the signaling reactions with *in vivo* measurements of protein copy number. Analysis of the model revealed two

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**Table 5.** Comparison of steady state levels of RR-P with and without CheA3 phosphatase activity.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Wild-type cells</th>
<th>No phosphatase</th>
<th>Fraction phosphorylated (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CheY3</td>
<td>30</td>
<td>88</td>
<td></td>
</tr>
<tr>
<td>CheY4</td>
<td>75</td>
<td>98</td>
<td></td>
</tr>
<tr>
<td>CheY6</td>
<td>64</td>
<td>99</td>
<td></td>
</tr>
<tr>
<td>CheB1</td>
<td>33</td>
<td>91</td>
<td></td>
</tr>
<tr>
<td>CheB2</td>
<td>40</td>
<td>96</td>
<td></td>
</tr>
<tr>
<td>Total RR pool</td>
<td>55</td>
<td>97</td>
<td></td>
</tr>
</tbody>
</table>

1 Lack of CheA3 phosphatase activity was simulated by setting $k_{15a} = k_{15b} = 0$ (Table 2).

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**Figure 4.** The predicted variation in levels of RR-P throughout a simulated chemotaxis response. Initially, CheA2 autophosphorylation in the polar cluster and the phosphorylation of CheA3 by CheA4 in the cytoplasmic cluster are occurring. To mimic attractant stimulation of the polar cluster, CheA2 autophosphorylation is turned off ($k_1 = 0$) at the point labelled (A). This causes a drop in RR-P levels and a new steady state is reached. (B) Subsequently, CheA2 autophosphorylation is turned back on and the system returns to its original steady state. At point (C), the phosphorylation of CheA3 by CheA4 is turned off ($k_2 = 0$) to mimic attractant stimulation of the cytoplasmic cluster. As a result of this, RR-P levels fall and a new steady state is reached. (D) Finally, phosphorylation of CheA3 by CheA4 is turned back on and the system returns to its initial steady state.

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interesting features of the signaling network, both of which rely on reversed phosphotransfer from RR-Ps to CheA. Firstly, rapid signal termination for all chemotaxis RR-Ps may be achieved by CheY₆ acting as a phosphate sink in addition to being the primary motor control protein (Figure 3A). Secondly, a novel phosphorelay involving CheB₂ appears to link the cytoplasmic and polar chemotaxis clusters (Figure 3B). Together, these two network features provide the bifunctional kinase/phosphatase, CheA₃, with the means to increase or decrease the concentration of all of the chemotaxis RR-Ps and therefore to regulate the output of the two chemosensory clusters.

CheY₆ is a phosphate sink for all of the chemotaxis RR-Ps
Phosphate sinks provide an alternative mechanism for dephosphorylating RR-Ps [51,52], instead of simply hydrolyzing the phosphoryl group (as in autodephosphorylation or phosphatase-assisted-dephosphorylation), the phosphoryl group is transferred to a HPK, which subsequently transfers it to the “phosphate sink” RR. The phosphoryl group is then hydrolyzed from the “phosphate sink” by either autodephosphorylation or phosphatase-assisted-dephosphorylation. The model presented in this study predicted that signal termination occurs rapidly in R. sphaeroides, with all RR-Ps dephosphorylating with half-times of less than ~7 s (Table 4). This is consistent with the observed stimulus response time of 1 s for R. sphaeroides [50], since with a decay half-time of ~7 s, CheY-P levels could fall by ~10% during 1 s, which, assuming that the R. sphaeroides motor is as unsaturative to changes in CheY-P levels as the E. coli motor [57], would be sufficient to give a significant change in motor rotation bias.

Prior to this study, it was known that CheY₅-P with the aid of its specific phosphatase, the bifunctional protein CheX₅, could dephosphorylate rapidly [48], however, the other RR-Ps were known to autodephosphorylate with half-times in excess of 36 s, with CheB₁-P taking over 4000 s. By removing each of the RR-Ps in turn from the model, we found that CheY₆ was acting as a “phosphate sink” for the other RR-Ps, since cells lacking CheY₆ showed much slower dephosphorylation rates for the remaining RR-Ps (Table 4 and Figure 3A). Furthermore, we showed that removal of the phosphatase activity of CheA₃ from the model increased the dephosphorylation half-times of all of the RR-Ps, indicating that the phosphatase activity is required for efficient operation of the CheY₆ phosphate sink and rapid signal termination. This phosphate sink role for CheY₆ is additional to its primary role as a direct regulator of flagellar rotation [39].

CheY₆ differs in several ways from the prototypical phosphate sink, CheY₁ from S. meliloti [51], CheY₆ directly controls flagellar motor rotation by binding FlIM [39,49], and has a dedicated phosphatase, in contrast, S. meliloti CheY₁ does not bind FlIM and appears to function only as a “phosphate sink”. Another fundamental difference is the rate of dephosphorylation; CheY₅-P dephosphorylates much faster than the autodephosphorylation rates of the RR-Ps for which it acts as a sink whereas S. meliloti CheY₁-P does not autodephosphorylate any faster than the motor binding RR, CheY₂-P, for which it is a sink. The S. meliloti sink does not need to dephosphorylate quickly because it does not directly affect flagellar rotation and so phosphoryl groups can be stored on it until autodephosphorylation occurs, in contrast, R. sphaeroides CheY₆ is a key regulator of flagellar rotation and therefore requires rapid signal termination.

A CheB₂ mediated phosphorelay connects the polar and cytoplasmic signaling clusters
As part of our interrogation of the model, we simulated attractant stimulation of the polar chemotaxis cluster by turning off autophosphorylation of CheA₂, while allowing phosphorylation of CheA₃ by CheA₄ to occur (Figure 4). Interestingly under these conditions, even though CheA₃-P cannot directly phosphorylate CheY₅, CheY₆, and CheB₁, the model predicted non-zero concentrations of these RR-Ps. This is the result of the action of a phosphorelay where phosphoryl groups from CheA₃-P (His) are transferred to CheB₂ (Asp) then to CheA₂ (His) and subsequently to either CheY₆, CheY₅ or CheB₁ (Asp) (Figure 3B). Direct testing of the in vivo importance of this phosphorelay is confounded by the dual role of CheB₂, firstly as a chemoreceptor methyltransferase and secondly as a potential intermediate in the phosphorelay. The methyltransferase activity of CheB₂ is required for normal chemotaxis and it is not possible to block the phosphorylation of CheB₂ by mutagenesis without impairing the control of this methyltransferase activity. It is therefore not known the extent to which this CheB₂ mediated phosphorelay operates in vitro; however, the model does include both in vitro kinetic preference data and in vivo protein expression levels, and this suggests that the phosphorelay may operate in vivo, allowing the cytoplasmic cluster to make a significant contribution to phosphorylation levels of the non-cognate RR-Ps: CheY₅, CheY₄ and CheB₁.

The model in this study does not include adaptation as this is a poorly understood process in R. sphaeroides, with little experimental data. However, it is possible that the adaptation pathway could act to reduce the elevated RR-P levels caused by a constant influx of phosphoryl groups to the polar cluster from the cytoplasmic cluster by modifying the polar receptors in such a way as to reduce the autophosphorylation rate of the polar kinase, CheA₂ i.e. the cell could adapt to constant signals from the cytoplasmic cluster. Although when cells are performing chemotaxis and swimming through gradients of chemoeffectors, signals from the cytoplasmic cluster will vary over time and will make a significant contribution to RR-P levels. The relative contribution of this phosphorelay to levels of CheY₅-P, CheY₆-P and CheB₁-P will be modulated by signals coming through the transmembrane chemoreceptors that directly control the rate of CheA₂ autophosphorylation and would be greatest when the autophosphorylation rate of CheA₂ is low and the rate of phosphorylation of CheA₃ by CheA₄ is high. These conditions could arise when cells are swimming up a gradient of a specific attractant which is sensed by the transmembrane chemoreceptors, while the metabolic state of the cell is worsening due to, for example, decreasing concentrations of an essential nutrient (for which there may not be a transmembrane chemoreceptor). Under such conditions, the increased rate of phosphorylation of CheA₃ by CheA₄, coupled with the CheB₂/CheA₃ mediated phosphorelay, could raise levels CheY₅-P, CheY₆-P and CheB₁-P, allowing cells to override their favourable response to the extracellular attractant and swim away from these unfavourable environments.

Numerous examples of other two-component systems employing phosphorelays have been described [58–61]; however, to the best of our knowledge this is the first example of a phosphorelay involving two distinct HPKs localized to different regions of the cell, and also, the first phosphorelay to be found in a chemotaxis signaling pathway. Given that over 50% of bacteria with any che genes have more than two cheAs [31,32,40], it seems likely that phosphorelays allowing communication between different CheA homologues could be involved in chemotactic signaling in a wide range of bacterial species.

Signal integration by the cytoplasmic cluster
The polar cluster senses extracellular signals while the cytoplasmic cluster will vary over time and will make a significant contribution to RR-P levels. The relative contribution of this phosphorelay to levels of CheY₅-P, CheY₆-P and CheB₁-P will be modulated by signals coming through the transmembrane chemoreceptors that directly control the rate of CheA₂ autophosphorylation and would be greatest when the autophosphorylation rate of CheA₂ is low and the rate of phosphorylation of CheA₃ by CheA₄ is high. These conditions could arise when cells are swimming up a gradient of a specific attractant which is sensed by the transmembrane chemoreceptors, while the metabolic state of the cell is worsening due to, for example, decreasing concentrations of an essential nutrient (for which there may not be a transmembrane chemoreceptor). Under such conditions, the increased rate of phosphorylation of CheA₃ by CheA₄, coupled with the CheB₂/CheA₃ mediated phosphorelay, could raise levels CheY₅-P, CheY₆-P and CheB₁-P, allowing cells to override their favourable response to the extracellular attractant and swim away from these unfavourable environments.
the cytoplasmic cluster. The kinase activity of the cytoplasmic cluster resides in CheA3 and the phosphatase activity resides in CheA4; both proteins have P5 (regulatory) domains and it is therefore likely that both activities will be regulated by environmental stimuli [48,62]. A stimulus that increases phosphatase activity would control sensitively the reduced levels of all RR-Ps since the action of the phosphatase would directly accelerate CheY6-P dephosphorylation leaving more unphosphorylated CheY6 to function as a phosphate sink for the other RR-Ps. Such a mechanism could allow the cytoplasmic cluster to tune or modulate signals coming from the polar cluster since increased phosphatase activity would lead to a general decrease in chemotaxis RR-P levels. In contrast, a stimulus that increased kinase activity at the cytoplasmic cluster would increase levels of all RR-Ps because CheA3-P would phosphorylate CheY6 and CheB2 directly; CheB2 would then shuttle the phosphoryl groups to CheA2 and from there onto the other RRs while phosphorylation of CheY6 would reduce its capacity as a phosphate sink resulting in a general increase in RR-P levels. Therefore the overall sensory output of the pathway depends critically on the relative activity of CheA3 and CheA4, and potentially provides a mechanism for signals about the metabolic state of the cell to modulate signals regarding the extracellular environment.

Materials and Methods

Mathematical model

Full details on the mathematical model are included in Text S1. Briefly, the law of mass action was applied to the reactions detailed in Table 1 to produce a system of non-linear ordinary differential equations (ODEs), which were solved using Matlab (MathWorks). The model was parameterized with data from the literature [39,43,45,48,63–65] and our own experiments as detailed in Tables 2&3. A parameter fit of the phosphotransfer rates between each kinase and RR (time course data) with that of a mathematical model describing the in vitro reactions was performed. A number of local, global and genetic algorithm optimisation procedures were employed (simulated annealing, Hooke and Jeeves, least squares, Levenberg-Marquardt, Nelder-Mead, steepest descent and the genetic algorithm) to obtain a robust set of parameters (Table 3).

Plasmids and strains

The plasmids and strains used are shown in Table S3. E. coli strains were grown in LB medium at 37°C. Where required, antibiotics were used at concentrations of 100 μg ml⁻¹ for ampicillin and 25 μg ml⁻¹ for kanamycin.

Protein purification

His-tagged and GST-tagged R. sphaeroides CheA, CheY and CheB proteins were purified as described previously [48]. Protein purity and concentration was measured as described [45]. Purified proteins were stored at −20°C.

Preparation of CheA3P1-32P

CheA3-P1 was phosphorylated using [γ-32P] ATP and CheA3, and purified as described previously [48]. The final preparation of CheA3-P1-32P was free of ATP and CheA3.

Detection of phosphotransfer from the response regulators to CheA2

Assays were performed at 20°C in TGMNKD buffer (50 mM Tris HCl, 10% (v/v) glycerol, 5 mM MgCl2, 150 mM NaCl, 50 mM KCl, 1 mM DTT, pH 8.0). CheA3P1-32P was used to phosphorylate the RRs, CheY6 and CheB2, in these assays because it is a good phosphodonor for these proteins and even after prolonged incubation (>1 hour) CheA3P1-32P does not act as a direct phosphodonor for CheA3 (Figure 3A); therefore any CheA2-P generated in these assays is due to phosphotransfer from RR-P to CheA3 rather than direct phosphotransfer from CheA3P1 to CheA2. 30 μM CheA3P1-32P was mixed with 5 μM CheA2 prior to the addition of 10 μM RR. Following the addition of RR, reaction aliquots of 10 μl were taken at the indicated timepoints and quenched immediately in 5 μl of 3 X SDS-PAGE loading dye (7.5% (v/v) SDS, 90 mM EDTA, 37.5 mM Tris HCl, 37.5% glycerol, 3% (v/v) β-mercaptoethanol, pH 6.8). Quenched samples were analyzed using SDS-PAGE and phosphorimaging as described previously.

Protein expression levels

Protein expression levels were measured in wild-type R. sphaeroides cells grown under microaerobic growth conditions using quantitative immunoblotting as described previously [65,67–69].

Supporting Information

Table S1 The effect of parameter variation on the simulation half-life of CheB1-P. Found at: doi:10.1371/journal.pcbi.1000896.s001 (0.09 MB PDF)

Table S2 The effect of parameter variation on the predicted levels of CheY4-P when CheA2 autoprophosphorylation is turned off (k2 = 0). Found at: doi:10.1371/journal.pcbi.1000896.s002 (0.09 MB PDF)

Table S3 Plasmids and bacterial strains used in this study. Found at: doi:10.1371/journal.pcbi.1000896.s003 (0.01 MB PDF)

Text S1 Mathematical modeling. Found at: doi:10.1371/journal.pcbi.1000896.s004 (0.02 MB PDF)

Author Contributions

Conceived and designed the experiments: MJT SLP PKM JPA. Performed the experiments: SLP. Analyzed the data: MJT SLP. Contributed reagents/materials/analysis tools: MJT SLP. Wrote the paper: MJT SLP PKM JPA.

References


